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Clearance kinetics and organ uptake of complement-solubilized immune complexes in mice

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SUMMARY

C3-bearing immune complexes were prepared by *in vitro* solubilization of BSA–anti-BSA complexes at equivalence. Sucrose density gradient analyses showed a size-heterogeneous population of solubilized complexes with a range of 7S to > 29S and a peak at around 19S. The presence of C3bi was demonstrated by precipitation with antibodies to C3c and to C3d and by binding to conglutinin. Immune complexes solubilized in two and three times antigen excess were selected as controls due to their size similarities with complement-solubilized complexes. Blood clearance curves were very similar for C3-bearing complexes and controls. At 1 hr, the percentage of injected material remaining in the circulation for complement-solubilized and two and three times antigen excess complexes were $29{\cdot}5 \pm 1{\cdot}3$, $30{\cdot}9 \pm 1{\cdot}7$ and $26{\cdot}1 \pm 2{\cdot}7$, respectively. Uptake by liver accounted for the majority of complement- and antigen-solubilized immune complexes removed from circulation. Although the uptake by the spleen was no more than one-tenth of the liver uptake, more complement-solubilized complexes than antigen-solubilized complexes were removed by this organ. The present data indicate that soluble immune complexes bearing C3 components and soluble immune complexes without C3 components, but of comparable size, are cleared from the circulation of mice at comparable rates. The mechanisms of clearance of these two populations of complexes, however, may differ.

INTRODUCTION

The presence of antigen–antibody complexes in the kidney has been well established in experimental models and in human glomerulonephritis. Subendothelial and mesangial immune deposits are thought to arise mainly from circulating immune complexes, whereas subepithelial immune deposits are primarily locally formed (reviewed by Mannik & Gauthier, 1984; Couser *et al.*, 1984). The formation of electron-dense deposits and persistence of immune complexes in the subendothelial areas or in the subepithelial area required the presence of precipitating antigen–antibody systems (Mannik, Agodoa & David, 1983; Agodoa, Gauthier & Mannik, 1983).

Miller & Nussenzweig (1975) demonstrated that complement can modify the size of immune precipitates *in vitro*. The incubation of immune precipitates with serum as a source of complement resulted in conversion of the precipitates to soluble immune complexes. Furthermore, complement inhibited immune precipitation when antigen and antibody were reacted in the presence of fresh serum, again resulting in soluble complex formation (Schifferli, Bartolotti & Peters, 1980). Although the classical and alternative complement pathway requirements were different, in both cases the resulting immune complexes contained C3 fragments (Fujita, Takata & Tamura, 1981; Schifferli, Woo & Peters, 1982; Naama *et al.*, 1984, 1985). The immune complexes containing fragments of C3 remained soluble, which has bearing on their deposition in glomeruli or other organs.

It would be of importance to determine how the presence of C3 fragments on immune complexes affects their fate in circulation as well as their ability to deposit in tissues. As a first step, we studied the clearance kinetics and organ uptake of C3-bearing immune complexes in mice and compared them to soluble complexes of similar size without C3.

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Abbreviations: Ab, antibody; Ag, antigen; anti-BSA, antibodies to BSA; anti-HSA, antibodies to HSA; BBS, borate-buffered saline; BBS^{2+}, BBS with Ca^{2+} and Mg^{2+}; BSA, bovine serum albumin; HSA, human serum albumin; PEG, polyethyleneglycol; SDG, sucrose density gradient; TCA, tricholoroacetic acid; VBS, veronal-buffered saline; VBS^{2+}, VBS with Ca^{2+} and Mg^{2+}.

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MATERIALS AND METHODS

Preparation of antigens, antibodies and soluble immune complexes

Human serum albumin (HSA) (E. R. Squibb, New Brunswick,

NJ) and bovine serum albumin (BSA) (Calbiochem, La Jolla, CA) were purified by ion exchange chromatography and gel filtered to remove aggregates.

Antibodies to HSA (anti-HSA) and BSA (anti-BSA) were isolated from hyperimmune rabbit serum by immunoadsorbents, rendered monomeric by gel filtration, and trace-labelled with ^{125}I by previously used methods (Mannik *et al.*, 1971). Antibodies to BSA were adsorbed with an agarose–HSA column to eliminate antibodies that might cross-react with HSA.

Immune complexes were prepared at equivalence. After overnight incubation at 4° the precipitates were washed twice in cold borate-buffered saline (BBS: 0·2 M borate, 0·15 M NaCl, pH 8·0), resuspended in BBS with Ca^{2+} and Mg^{2+} (BBS^{2+}: 0·15 mM Ca^{2+} and 0·5 mM Mg^{2+}) and passed through a 27-gauge needle to break up large particles.

In order to establish the best conditions for complement-mediated solubilization of immune complexes, varying amounts of serum and complexes were mixed using fresh human serum or serum after storage at −70° for periods less than 2 months. As a control, heat-inactivated (40 min at 56°) serum was used. Immune complexes and complement were incubated for 2 hr at 37°, centrifuged for 10 min at 3000 r.p.m, and precipitates and supernatants separated and assayed for radioactivity.

In order to obtain antigen-solubilized immune complexes, immune precipitates were prepared, washed, and amounts of antigen were added to reach final concentrations of two, three, four and five times antigen excess. These mixtures were dialysed overnight against acetate buffer, pH 3·5, and for 8 hr against phosphate-buffered saline, pH 7·2. The precipitates and supernatants were separated by centrifugation and assayed for radioactivity.

Complement-solubilized immune complexes were gel filtered on Sephacryl S-200 (Pharmacia Fine Chemical AB, Uppsala, Sweden) for some experiments.

Sucrose density gradient analysis

The size of the different immune complex preparations as well as the monomeric nature of the protein preparations were characterized on 10–50% linear sucrose density gradients (SDG) in BBS, run at 37,000 r.p.m. at 4° for 16 hr in a SW 41 Ti rotor and a Beckman L2-65B ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The tubes were loaded with 300 μl of a 1:2 dilution of the immune complexes in BBS^{2+}. Fractions were collected and analysed for radioactivity. The bottom ends of the centrifuge tubes were also analysed and their radioactivity referred to as the pellet.

Analysis of C3 components in complement-solubilized immune complexes

Specific rabbit antisera against the C3c and C3d fragments of the C3 molecule (Nordic Immunological Laboratories, El Toro, CA) were used to precipitate complex-bound C3. Antisera were added in sufficient amount to combine with all the antigen present in selected pools of SDG fractions. Controls included normal rabbit serum and rabbit anti-transferrin. Mixtures were incubated for 1 hr at 37° and overnight at 4°. After adding a constant amount of BBS to increase the volume, tubes were centrifuged for 15 min at 3000 r.p.m. and exactly half the supernatant and the precipitate plus remaining supernatant were counted to calculate the percentage of radiolabelled antibody in the complexes that had bound C3 components.

Conglutinin binding experiments

Bovine conglutinin was purified from bovine serum (Maire, Barnet & Lambert, 1981). A conglutinin column was prepared by coupling the purified material to polymethylmethacrylate beads (Degalan V 26, Accurate Chemical and Scientific Corp., Westbury, NY). Complement-solubilized complexes were applied and eluted from the column as described by Casali & Lambert (1979). Briefly, one volume of solubilized complexes was precipitated with one volume of 5% polyethyleneglycol (PEG) in veronal-buffered saline (VBS) containing Ca^{2+} and Mg^{2+} (VBS^{2+}). The collected precipitate was dissolved in VBS^{2+} and applied to the column. Elution was carried out with 0·02 M EDTA–VBS. The eluted fractions were dialysed against VBS^{2+}.

In vivo *experiments*

C57BL/6J female mice of approximately 20 g were used. Injections of 0·3–0·5 ml samples contained 20–25 μg of antibody in immune complexes. For clearance kinetics, retro-orbital plexus blood samples of 20 μl were drawn in heparinized tubes at 1, 2, 4, 6, 8, 10, 15 and 30 min, and 1, 2, 4, 8, 12, 24, 48, 72 and 96 hr. Samples were added to 1 ml of 0·1 M HCl and proteins were precipitated with 0·5 ml of 22·5% trichloroacetic acid (TCA). After centrifugation, the pellet and supernatant were separated and assayed for radioactivity. The percentage of TCA precipitable radioactivity remaining at a given time-point was calculated from the counts per 20 μl and the counts at time zero. The latter values were extrapolated for each mouse from the first three data points.

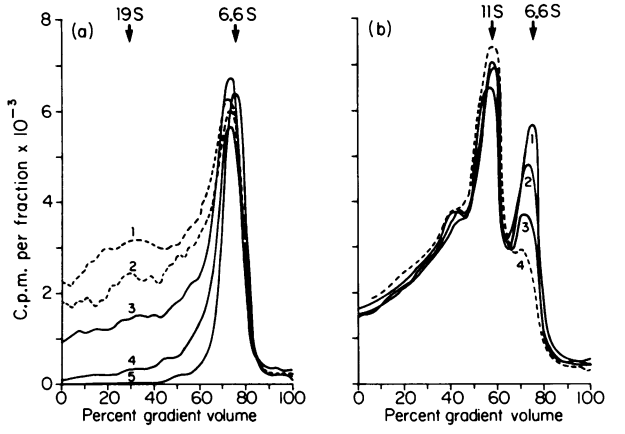
Organ distribution of immune complexes was determined in another set of animals injected with the same preparations and killed at 10 and 30 min and 1, 12, 24 and 48 hr, collecting as much as possible of their circulating blood. Individual organs, carcasses and skin were counted, and the radioactivity within every organ expressed as a percentage of injected dose.

RESULTS

Characterization of solubilized immune complexes

Based on preliminary experiments, we prepared complement-solubilized immune complexes by mixing 300 μl of human serum and 20 μg antibody in BSA–anti-BSA precipitates, yielding solubilized immune complexes with about 18 μg of antibodies. The size of complement-solubilized immune complexes was determined by SDG ultracentrifugation. Immune complexes incubated with either heat-inactivated serum or buffer were included. The supernatant of these controls contained a peak that sedimented the same as monomeric anti-BSA (Fig. 1a). This probably represents low-affinity antibodies detached from the lattice during the incubation period at 37°. With increasing amounts of fresh serum, the supernatant contained immune complexes with varying size, including monomeric 6·6S material and complexes heavier than 29S. A peak of approximately 19S material was observed.

Because our purpose was to compare the *in vivo* behaviour of soluble immune complexes with or without complement, we also prepared soluble complexes containing different amounts of antigen excess. Appropriate amounts of antigen were added



Sample	Serum Used	Solubilized IC (%)	^{125}I in Pellet (%)
1	300μl Fresh	94	7·9
2	150μl Fresh	90	11·2
3	100μl Fresh	70	14·5
4	50μl Fresh	35	<1
5	300μl Heated	22	<1

Sample	Times Antigen Excess	Solubilized IC (%)	^{125}I in Pellet (%)
1	2	58	16·8
2	3	91	13·3
3	4	94	8·4
4	5	93	4·0

Figure 1. Sucrose density gradient ultracentrifugation patterns of immune complexes solubilized by complement (a) or antigen excess (b). (a) Twenty μg of antibody in immune precipitates were solubilized with the indicated amounts of serum. (b) Immune complexes were formed at equivalence and then dissociated with indicated amounts of antigen excess. The tables below the patterns indicate the percentage solubilized by complement in the third column, and the fourth column shows the percentage radioactivity in the pellet of total applied to the gradient.

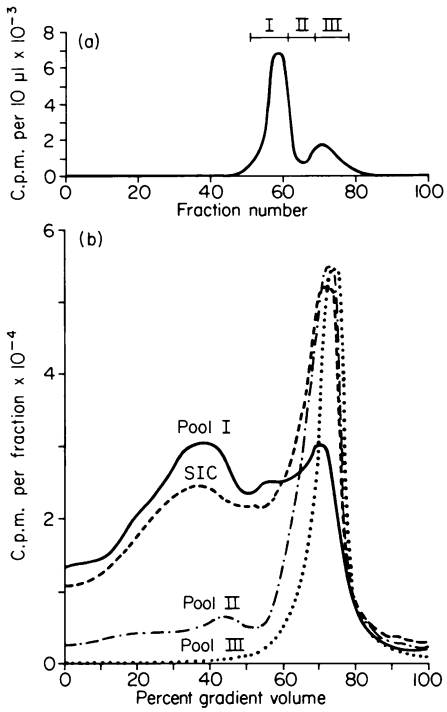


Figure 2. Gel filtration of complement-solubilized immune complexes and sucrose density gradient ultracentrifugation of the resulting fractions. (a) Sephacryl S-200 gel filtration profile of complement-solubilized immune complexes. Three pools were made and analysed by sucrose density gradient ultracentrifugation (b) and compared to the unfractionated complement-solubilized immune complexes (SIC).

to yield final complexes with two, three, four or five times antigen excess. With the exception of immune complexes containing two times antigen excess, the percentage of solubilization was, in all cases, 80–90% of the starting material. Two major peaks, one of free antibody (6·6S), which decreased with increasing amounts of antigen excess, and a 11S peak of complexes, appeared in all samples (Fig. 1b).

Gel filtration of complement-solubilized immune complexes on a Sephacryl S-200 column was carried out to separate complexes from monomeric antibodies. Two peaks were obtained and pooled (Fig. 2). Pools I, II and III represent heavy immune complexes, intermediate-small complexes and monomeric antibodies, respectively. These pools were concentrated and analysed on SDG to assess their size. The patterns obtained revealed that Pool I was composed mostly of a heterogeneous population of complexes that further dissociated to contain monomeric antibodies (Fig. 2b). Pool II contained some heavy complexes and a major peak of monomeric antibodies, and Pool III consisted mainly of monomeric antibodies with a heavier shoulder, indicating the presence of material other than antibodies.

Presence of C3 components on the complement-solubilized complexes

SDG fraction of complement-solubilized complexes were analysed for the presence of C3 by precipitation with rabbit antibodies to C3c or C3d. Normal rabbit serum, rabbit anti-transferrin and buffer were used as controls, which gave an insignificant precipitation in all cases. As expected, the heterogeneous population of immune complexes contained C3. It was of note that over 20% of Fraction 7 was precipitated with both antisera, indicating the presence of small-latticed (e.g. Ag_1 Ab_1) complexes or antibody molecules with covalently bound C3 (Fig. 3). Because the maximal precipitation with antibodies to C3 was around 40% in the diluted SDG fractions (Fig. 3), the presence of C3 on complement-solubilized complexes was also analysed after fractionation by gel filtration on S-200 (Fig. 2a). From Pool I, 59% of radioactivity was precipitated with anti-C3c, and 89% with anti-C3d. For Pool II the values were 21% and 87%, respectively.

Purification of complement-solubilized immune complexes was performed on a conglutinin column. The material that dissolved after 2·5% PEG precipitation was applied to the conglutinin column. The fall-through fraction contained 34% of the applied radioactivity. The fraction eluted with EDTA-buffer contained 66% of radioactivity and represented the C3bi-bearing population of solubilized immune complexes.

Clearance kinetics of complement-solubilized immune complexes as compared to antigen excess soluble immune complexes

Blood clearance kinetics of complement-solubilized immune complexes were performed in mice and compared to complexes solubilized by two- and three-fold antigen excess. The latter were selected because of their size similarities with complement-

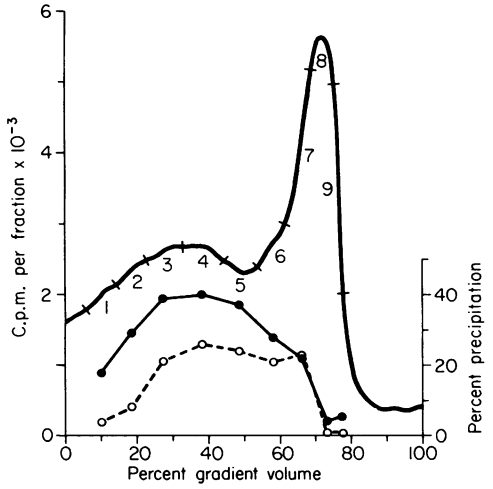


Figure 3. Presence of C3 on complement-solubilized immune complexes. Fractions of sucrose density gradients of solubilized immune complexes (——) were precipitated with anti-C3c (o---o) or anti-C3d (●——●).

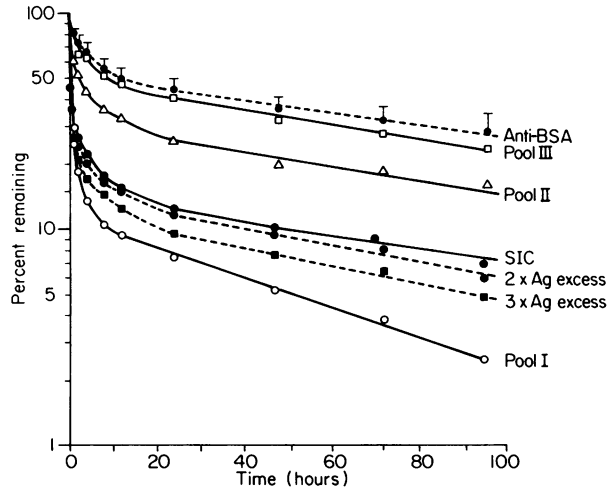


Figure 4. Removal of solubilized immune complexes from circulation. Mice were injected with complement-solubilized immune complexes (●——●) or with two (●---●) or three (■---■) times antigen excess soluble complexes. Monomeric anti-BSA (●---●) and complement-solubilized immune complexes separated into three pools by gel filtration as indicated in Fig. 2 were also injected: (o——o) Pool I; (△——△) Pool II; (□——□) Pool III. Each data point represents the mean for three mice. The ±SD range is plotted only for the anti-BSA, but those for all other preparations were comparable.

solubilized immune complexes. For comparison, clearance kinetics of S-200 fractionated, complement-solubilized complexes (Pools I, II and III) and monomeric rabbit anti-BSA were also studied (Fig. 4). Pool I, containing the heavy component of solubilized immune complexes, cleared from circulation faster than the unfractionated preparation and faster than the antigen-solubilized complexes; 88·5% of Pool I was rapidly removed from the circulation, as reflected by the y intercept of the slow exponential component. Pool II, which contained monomeric antibodies with and without C3 and small-latticed immune complexes, cleared at a slower rate; 68% of this material was removed rapidly or equilibrated with extravascular space. Removals of anti-BSA and Pool III were very similar, confirming the monomeric antibody nature of the latter. By 1 hr, the remaining percentage of injected radiolabelled material in the circulation for unfractionated complement-solubilized immune complexes, Pools I, II and III and monomer were 29·5±1·3, 24·8±0·6, 60·3±4·6, 75·5±6·2 and 81·0±4·4, respectively.

When unfractionated, complement-solubilized complexes were compared with two and three times antigen excess soluble complexes, very similar clearance profiles were obtained (Fig. 4). This is also reflected by the percentages of material remaining in the circulation at 1 hr for the complement-solubilized, two and three times antigen excess complexes were 29·5±1·3, 30·9±1·7 and 26·0±2·7, respectively.

Tissue distribution of complement-solubilized immune complexes

These experiments were conducted to determine the influence on tissue deposition of the presence of complement fragments on soluble complexes. Uptake by the liver accounted for removal of the majority of circulating complement- and antigen-solubilized immune complexes. In the initial hepatic uptake, no statistically significant difference was observed between the two preparations of immune complexes. At 12 hr and later, however, more radioactivity of the complement-solubilized complexes remained in the liver than of the antigen-solubilized complexes (Table 1). This suggested that the degradation of the complement-solubilized complexes was slower than the degradation of antigen-solubilized complexes, as confirmed by the lower levels of TCA-soluble radioactivity in the blood of mice given the

Table 1. Organ distribution of complement- and antigen excess-solubilized immune complexes

Preparation	Interval†	Liver	Spleen	Kidney
CSIC‡	10 min	56·3±4·7§	5·7±0·9	1·5±0·2
3×Ag IC¶		50·2±5·2	3·3±0·7*	1·1±0·3
CSIC	30 min	57·9±5·7	5·1±0·9	1·2±0·2
3×Ag IC		51·6±5·0	3·2±0·7*	1·0±0·1
CSIC	1 hr	51·3±9·9	3·5±0·8	0·9±0·3
3×Ag IC		47·0±9·5	2·1±0·7	1·0±0·2
CSIC	12 hr	10·2±1·6	1·1±0·1	0·8±0·2
3×Ag IC		2·3±0·4**	0·3±0·1*	0·4±0·1
CSIC	24 hr	1·9±0·4	0·8±0·2	0·4±0·2
3×Ag IC		0·9±0·2*	0·2±0·0*	0·2±0·0
CSIC	48 hr	1·2±0·1	0·8±0·3	0·3±0·1
3×Ag IC		0·5±0·2**	0·1±0·1**	0·1±0·0

The statistical analysis between complement-solubilized and antigen-excess immune complexes for each organ was done using the t-test: *$P<0·05$; **$P<0·02$.

†Time after injection of radioactive material.

‡Complement-solubilized immune complexes.

§Values expressed as a percentage of injected dose. Each value = mean ± one SD of three mice/experiment.

¶Soluble immune complexes prepared at three times antigen excess.

complement-solubilized *vs* control complexes. Although the uptake of immune complexes by the spleen was relatively small compared to the liver, the uptake of complement-solubilized immune complexes was higher than that of the antigen-solubilized complexes (Table 1). No differences were found in the amount of the two preparations in kidneys. The percentages were small, and the counts were not corrected for blood remaining in the kidneys. Therefore, no conclusions can be drawn from these data on the renal deposition of these materials. No preferential deposition in heart, lungs, digestive tract or skin was apparent between complement- and antigen-solubilized complexes (data not shown).

DISCUSSION

The studies described herein were designed to examine the fate of circulating C3-bearing immune complexes in mice. C3 is the central component of both complement pathways, and complexes containing C3 breakdown products result from complement solubilization of immune precipitates or complement inhibition of immune precipitation (Miller & Nussenzweig, 1975; Schifferli *et al.*, 1980). The presence of C3 fragments on the solubilized complexes was proven by the ability of antisera to C3c and C3d to precipitate these complexes. As pointed out, 89% of the large, complement-solubilized complexes contained C3d antigen. In diluted solutions of sucrose density gradient fractions, the percentage of complexes precipitated with antibodies to C3d was around 40%. These findings indicated that the majority of the solubilized, large immune complexes contained C3d. As reflected by the binding to conglutinin, in 66% of the complexes the C3 was present as C3bi. Therefore, these preparations were adequate to examine the effect of C3 fragments bound to immune complexes on their clearance from circulation and organ uptake.

In order to compare complement-solubilized complexes with complexes of comparable size without C3 fragments, soluble complexes were prepared at different times of antigen excess. Two and three times antigen excess immune complexes showed a comparable, but not identical, size distribution pattern. When injected into mice, the clearance kinetics of both types of complexes were comparable. The participation of mouse complement in the clearance of the antigen-solubilized complexes is unlikely since in previous investigation extensive depletion of complement caused no change in the clearance of comparable complexes (Bockow & Mannik, 1981). The heaviest population of complement-solubilized complexes separated by gel filtration (Pool I) cleared from the circulation at the fastest rate. It is worthy of note that even this preparation was not cleared with a single exponential component, but the slowest component possessed a y intercept of 11·5%. This slow component might be explained by re-equilibration of the complexes after isolation, since a significant amount of radioactivity was found in the peak corresponding to monomeric antibodies (Fig. 2b) that could not have been present after gel filtration (Fig. 2a).

Two previous investigations have examined complement-solubilized immune complex clearance from circulation and uptake in tissues of mice (Malasit, Bartolotti & Humphrey, 1983; Takahashi *et al.*, 1985). In the first study, complement-solubilized immune complexes were compared to insoluble complexes, and therefore the faster clearance from circulation and increased uptake of the latter in lungs and liver could have been anticipated. In the second study, rabbit antibodies to ovalbumin were used for the preparation of immune complexes, complement solubilization was achieved with mouse serum, and the clearance kinetics and tissue uptake of the solubilized complexes were compared to immune complexes made at five-fold antigen excess. The dose of antibodies in the complexes was 1·5 μg. The disappearance of the complement-solubilized complexes from circulation was faster than those made in antigen excess. The lattice of the latter, however, was not characterized, and comparative uptake by liver or other organs was not described. The uptake of complement-solubilized immune complexes by the liver, however, was comparable to the results obtained by ourselves, i.e. about 50% at 20 min. Uptake of complement-solubilized complexes was shown to be by Kupffer cells in the liver. Furthermore, it has been documented that uptake of these complexes by mouse macrophages was mediated mainly by CR3 (C3bi receptor) and, to a lesser degree, by Fc receptors, as determined by appropriate inhibitors (Takahashi *et al.*, 1985). As pointed out, Takahashi *et al.* (1985) solubilized immune precipitates with mouse serum as a source of complement, whereas we used human serum. It is worthy of note that the same monoclonal antibodies blocked the function of mouse and human CR3, even though the rosetting of mouse macrophages with red cells containing human complement components was less than those containing mouse complement components (Beller, Springer & Schreiber, 1982). The data described by ourselves clearly show that the liver is the major organ for removal of circulating immune complexes carrying C3 fragments, obtained by complement-mediated solubilization of immune precipitates. The rates of uptake of these complexes and of antigen-solubilized complexes were comparable. As already discussed, others have presented evidence for the involvement of CR3 in hepatic uptake of complexes containing C3bi (Takahashi *et al.*, 1985). Species differences of complement components may have caused some differences in results, but the hepatic uptake is quite comparable for immune complexes containing mouse or human complement components.

In view of available evidence, it remains unclear what role the Fc receptors play in the clearance of complement-solubilized complexes that actually contain C3 components. Controversial reports exist regarding the exposure of the Fc portion of the antibody in the complexes after complement solubilization. Malasit *et al.* (1983) concluded that the Fc portions were obscured by attached C3; Takahashi *et al.* (1985) reported that the solubilization process inhibited the binding of the resulting complexes to the Fc receptor, and Scharfstein *et al.* (1979) found that these complexes lost their binding affinity to staphylococcal protein A, suggesting that the Fc is covered by complement components. In contrast, Schifferli & Peters (1983) showed a very efficient binding of staphylococcal protein A to complement-solubilized complexes or complexes formed in the presence of serum. Furthermore, two studies have shown that C3 attaches to complement-solubilized complexes through the Fd region of IgG (Gadd & Reid, 1981; Takata, Tamura & Fujita, 1984).

A very important question that remains to be answered is whether circulating C3-bearing complexes can deposit in tissues and cause tissue damage, or are destined for disposal without the ability to form tissue deposits. C3b is covalently bound to the antibody and possibly to the antigen (Takata *et al.*, 1984),

which could account for the observed lack of precipitability of complement-solubilized immune complexes. Therefore, it is quite possible that the C3-bearing complexes generated by complement solubilization or by inhibition of precipitation could no longer rearrange and condense. These properties, however, have been shown to be necessary for large lattice formation and persistence of immune deposits in glomeruli (Mannik *et al.*, 1983; Agodoa *et al.*, 1983). Several clinical investigations have shown that low levels of C3 imply decreased complement solubilization or inhibition of precipitation capacity in sera of patients with systemic lupus erythematosus, and that this impairment parallels the severity of renal disease (Aguado *et al.*, 1981; Schifferli *et al.*, 1981; Baatrup *et al.*, 1983, 1984).

The next logical step in this line of enquiry is to prepare sufficient quantities of soluble immune complexes containing covalently bound C3 components to determine if these complexes can form electron-dense and persistent immune deposits in glomeruli.

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